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Research Article

Geohelminths and Bacteria Associated with Cooked, Unshelled African Walnuts (*Tetracarpidium conophorum*)

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ABSTRACT

Walnuts (*Tetracarpidium conophorum*) are globally recommended for their several health benefits. Here, we report on the geohelminths and bacteria associated with the cooked walnut shells. Cooked unshelled walnuts were purchased from Mile III Market in Port Harcourt, Nigeria in the month of September 2023, and examined for geohelminth and bacterial contamination using standard techniques. Geohelminths were identified using standard keys while bacteria were identified using their characteristics observed from morphological and biochemical tests. Isolated bacteria were also enumerated and calculated using appropriate formula. One geohelminth species (*Strongyloides stercoralis*) was encountered. Counts of total heterotrophic bacteria was $1.50x10^6$CFU/g; total coliform was $2.00x10^4$; faecal coliform was $1.30x10^4$, *Salmonella*/*Shigella* was $8.9x10^3$; and *Staphylococcus* species was $1.7x10^3$CFU/g. Bacterial species of the following genera were identified; *Bacillus*, *Staphylococcus*, *Salmonella*, *Shigella*, *Pseudomonas*, *Enterobacter*, *Escherichia*, *Micrococcus*, *Acinetobacter* and *Klebsiella*. Walnuts are commonly sold in a cooked, unshelled state. The presence of the geohelminth and bacterial species on the shells entails health concerns in persons cracking the shells in their teeth. It is hence, suggested that cooked, unshelled walnuts should be washed in saline water before cracking and consumption.

Keywords: African walnuts, geohelminth, parasites, *Strongyloides*, *E. coli*, bacteria, public health.

Introduction

The African walnut (*Tetracarpidium conophorum*) belongs to the family Euphorbiaceae (Adetunji *et al*., 2021). These nuts are widely recognized for their health benefits, whether eaten as whole nuts or by use of the extracted oil (Adetunji *et al*., 2021; Song *et al*., 2022). They are known to have very high antioxidant properties and have been reported to be effective in controlling chronic diseases including neurological, cardiovascular and inflammatory diseases, and enhancing fertility in man as well as improving brain function (Adetunji *et al*., 2021; Binici *et al*., 2021).

They have also been reported to have a prebiotic effect on the gut microbiota by increasing the population of beneficial bacteria, such as *Lactobacillus* sp. which assist in the breakdown of indigestible fibers into butyrate (Bamberger *et al*., 2018).

Pathogens can infect walnut kernels, reducing their nutritional composition and causing post-harvest losses. For instance, in India, Gulam *et al*. (2023) isolated *Dothiorella gregaria* from walnut kernels sold in markets. These authors reported significant reduction in the polyphenol content, and hence antioxidant value of infected kernels. Other nuts are also vulnerable to bacterial and fungal contamination (Berthold-Pluta *et al*., 2021; Mirabile *et al*., 2021) which can lead to diseases in children and immunocompromised individuals. In addition, the cooked, unshelled nuts may also harbour parasites and microbes that can also cause infection in man.

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In Nigeria, edible walnuts are usually sold as cooked, un-shelled nuts, usually wet in wheelbarrows or on trays which are exposed to flies and rodents. Intending buyers also handle the nuts thereby unknowingly transferring pathogens from infected hands to the nuts. In most places in Nigeria, consumers buy these exposed nuts and use their teeth to crack them before un-shelling and consuming the kernels. In this process, parasites and microbes can be introduced into the mouths of these consumers, causing various infections.

Research on the contamination of walnut shells by parasites or microbes is scanty. Jahanban-Esfahlan *et al.* (2020) reported on the use of walnut shells in the removal of hazardous compounds. Some researchers have reported the use of walnut hulls for the treatment of parasitic intestinal worms (Wenzel *et al*., 2017) and use of the shells as anticancer and antibacterial agents (Fordos *et al*., 2023). However, there is paucity of information on parasitic helminths or microbes on the cooked, unshelled nuts. This research therefore examined market-derived cooked unshelled African walnuts (*Tetracarpidium conophorum*) for geohelminths and bacterial contaminants as an issue of public health importance.

Materials and Methods

Sample collection

The samples of African walnuts (*Tetracarpidium conophorum*) were purchased from traders retailing the cooked unshelled nuts in wheelbarrows at Mile III Market, Port Harcourt, Nigeria. Samples were aseptically collected from the traders into sterile zip-lock packs and immediately transported to the parasitology laboratory of the Department of Animal and Environmental Biology and laboratory of the Department of Microbiology, Rivers State University, Port Harcourt, Nigeria for parasitological examination and microbiological analysis respectively.

Parasitological Examination of Walnuts

Two hundred grams (200g) of the walnut shell samples were weighed using a digital weighing balance (Camry Model EK5350) and kept in previously washed containers. The unshelled nuts were washed in 200ml of physiological saline. The solution was sieved into another container using a hand-held sieve of about 1mm mesh size.

The filtrate was kept over-night to sediment after which the supernatant was decanted. The deposit was shaken vigorously and 12ml was poured into centrifuge bottles until all the sediment was used up. These were fixed into a bucket centrifuge (model: TD4A) which was set to spin at 3000rpm for 15minutes.

After centrifuging, the supernatant of each tube was decanted. The sediment from all tubes were then mixed together and examined under the microscope using x10 and x40 objective lens.

Parasite identification was aided by keys from Cheesebrough (2006a) and the photograph taken by means of a Nikon digital camera attached to the objective lens of a compound microscope.

Bacteriological Examination of Walnuts

Sterilization of equipment and media

All glass wares used during the course of this research were carefully washed with detergent, rinsed with tap water and finally rinsed with deionized water. Media were sterilized in an autoclave at 121^{o}C at 15 psi for 15 minutes. Equipment easily denatured by heat (example, plastics), were sterilized using 99.9% ethanol. Normal saline was used as diluent at a concentration of 0.85%. Nutrient Agar (NA) was used for the determination of Total Heterotrophic Bacteria (Cheesbrough, 2006b).

Cultivation and enumeration of bacterial isolates

Ten grams (10g) of walnut shell samples were weighed using a weighing balance and homogenized in 90ml of normal saline. Subsequently, a ten-fold serial dilution was carried out and an aliquot from appropriate dilutions was inoculated onto duplicate Petri plates of various solidified agar medium. Inoculum was spread-plated on media such as nutrient agar for the growth of total heterotrophic bacteria (THB), MacConkey agar for total coliform, Eosin Methylene Blue (EMB) agar for total faecal coliform and incubated at 44.5^{o}C, *Salmonella-Shigella* agar for total *Salmonella/Shigella*, and on Manitol salt agar for total Staphylococci (Cheesbrough, 2005). Except for the EMB cultured agar plates, other cultured plates were incubated at 37^{o}C for 24hours.

Bacterial colonies that grew on the respective culture media plates were counted and the mean expressed as colony forming units per gram (CFU/g) of the walnut

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Shell samples (Prescott *et al*., 2005), using the formula below;

$$\text{CFU/g} = \frac{\text{number of colonies}}{\text{Dilution x volume plated}}$$

Maintenance of pure culture

Discrete colonies from the respective media were sub-cultured unto freshly prepared nutrient agar plates and incubated at 37ºC for 24hours in order to obtain pure culture. The pure cultures of the organisms were obtained and preservation of the isolates was done according to the method of Ali and Naseem (2011).

Biochemical characterization of the pure cultures

Gram staining

The bacterial Isolates were Gram stained to determine their response to stains. A thin smear was made on a clean microscopic glass slide; air dried and heat fixed on the slide by passing it over a flame. The smear was then stained in the following order:-

1. Crystal violet for 30 to 60 seconds and rinsed with water,
2. Lugol's iodine for 60 seconds and rinsed with water,
3. Alcohol 5 to 10 seconds and rinsed with water
4. Safranin 60 seconds and rinsed with water

Smear was allowed to dry and then viewed under light microscope with oil immersion objective lens.

Motility Test

Medium was prepared according to manufacturer's specification dissolving 2.8g of nutrient agar in 100mls of distilled water. 10ml of the semi-sold agar was dispensed into test tubes and tubes were autoclaved at 121°C for 15 minutes. It was left to set in a vertical position. Inoculation was made with a straight sterile wire making a single stab down the centre of the tube to about half the depth of the medium and incubated at 37°C for 24 hours.

Motile bacteria were characterized by giving diffuse hazy growths that spread throughout the medium rendering it slightly opaque. For non-motile bacteria that yield motile variants, a discrete line of growth was formed along the stab and there was a diffused outgrowth that fanned out from one.

Catalase test

Hydrogen peroxide solution was transferred into various test tubes and flamed bent glass rod was used to inoculate some colonies of the respective 24 hour's pure culture into the test tubes containing the hydrogen peroxide separately. The contents of the test tubes were observed for effervescence (gas bubbling).

Urease test

A loopfull of 24 hours pure culture was inoculated into a prepared and sterilized urea broth (the urea broth is yellow-orange in colour). Change in colour, from yellow-orange to purple-pink indicates positive for urease, an enzyme that hydrolyzes urea into ammonia.

Oxidase test

Two to four drops of newly prepared oxidase reagent were placed on the piece of a filter paper placed in petri dish. A flame bent glass rod was used to pick a colony of the isolates and made a smear on the filter paper and then allowed for about 10 seconds.

A deep purple colouration within 10 minutes indicates the presence of an enzyme oxidase which converts the reagent rapidly to that deep purple colour.

Indole test

Colonies of 24 hours pure culture were inoculated into test tubes containing 5 ml of sterilized tryptophan broth and incubated at 37ºC for 48 hours. After incubation, Indole was tested by addition of 0.5 ml of Kovac's reagent to test tubes.

The mixture was shaken vigorously and observed for the development of red colour on the surface layer within 10 minutes.

Coagulase test

A smear was made on the both end of clean glass slide with sterile saline. A flamed bent glass rod was used to pick a colony of each isolate and emulsified in each of the drops of saline to make thick smear.

Plasma was added to one of the smear using wireloop and mixed vigorously while on the other end no plasma was added to the second smear and this was used as a control. The clumping of the organisms was observed within 10 seconds.

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Citrate utilization

Simon citrate agar was prepared in accordance with the manufacturer' instruction: 25 grams was dissolved in 1 litre. Then dispensed into test tubes and sterileze by autoclaving at 15 psi (121°C) for 15 minutes and allowed to solidify at slant position. This test was used to determine the ability of an organism to utilize citrate present in Simon's medium as a main source of carbon for growth. The isolates were inoculated using inoculating loop and incubated at 37°C for 48 hours.

The content of the test tubes were examined and the development of a blue colouration as against the original green colour of the medium indicates positive result.

Methyl red test

Glucose phosphate broth was prepared, 5ml was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 minutes at 15 psi. After cooling, the isolate was inoculated into the broth with a wire loop and incubated for five days at 37°C for 24 hours. After incubation, 5 drops of 0.04% solution of alcoholic methyl red were added into each test tube and mixed vigorously. The contents were examined immediately for the development of bright red colouration.

Voges – Proskauer Test

After sterilization, the medium was allowed to cool and the isolate was inoculated into Glucose phosphate broth and incubated for four days at 37°C. After incubation, 1.5 ml of 5% alcoholic alpha napthtol and 0.5 ml of 40% aqueous KOH were added.

The test tubes were shaken thoroughly and allowed to stand for 5 minutes. The content was examined for the development of pink or red colour.

Sugar Fermentation Test

The sugars tested were glucose, sucrose, fructose, maltose, and lactose. 1g of sugar was added into 80ml of peptone water and stirred thoroughly to solubilize the sugar, after which 20ml of 0.2% (w/v) methyl red indicator was added to the sugar-peptone water solution.

Ten millimeter (10ml) of the sugar-peptone water solution was dispensed into test tubes containing inverted Durham's tubes and autoclaved at 121°C for 15 minutes. The test tubes were allowed to cool, inoculated with an overnight growth culture (24 hours old culture). The tubes were incubated at 37°C for 24 hours. An orange colour showed a positive result for both fermentation and oxidation and presence of bubbles in the Durham's tubes indicated gas production.

Results

The result ofparasitological examination and microbiological analysis (morphological and biochemical characteristics) of the walnut shell samples from this research showed the presence of one geohelminth and ten bacterial species.

The African walnut (*Tetracarpidium conophorum*) shells were found to be infested with three larvae of the geohelminth parasite identified as *Strongyloides stercoralis* (Plate 1). No other geohelminths were encountered.

The result of counts of the various groups of bacteria and the identified bacterial species isolated from African walnuts (*Tetracarpidium conophorum*) in Port Harcourt are as shown in Table 1 and Table 2 respectively.

The count of total heterotrophic bacterial was $1.50x10^6$ CFU/g of walnut shell, while the total coliform count was $2.00x10^4$ CFU/g. The counts for faecal coliform, *Salmonella*/*Shigella* and *Staphylococcus* species were $1.30x10^4$ CFU/g, $8.9x10^3$ CFU/g and $1.7x10^3$ CFU/g, respectively. The decreasing order of the counts of these bacterial groups was as follows: Total heterotrophic bacteria > total coliform > faecal coliform > *Salmonella*/*Shigella* > *Staphylococcus*.

Bacterial identification following morphological and biochemical tests conducted revealed the presence of the following bacteria species: *Bacillus* sp., *Staphylococcus* sp., *Salmonella* sp., *Shigella* sp., *Pseudomonas* sp., *Enterobacter* sp., *Escherichia coli*, *Micrococcus* sp., *Acinetobacter* sp. and *Klebsiella* sp. (Table 2).

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Plate 1: *Strongyloides stercoralis* isolated from Walnuts (*Tetracarpidium conophorum*) shells (Scale: 0.01mm) (Photo by Amuzie, C.C., 2023)

Table 1: Bacterial counts (CFU/g) of African walnuts sold in Mile III Market, Port Harcourt

Sample	Total Heterotrophic Bacteria	Total Coliform	Feacal Coliform	*Salmonella/ Shigella*	*Staphylococcus*
Walnut	$1.50x10^6$	$2.00\ x10^4$	$1.30\ x10^4$	$8.9\ x10^3$	$1.7\ x10^3$

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Table 2: Morphological and Biochemical Characteristics of Bacterial Isolated from African Walnuts (*Tetracarpidium conophorum*)

Isolate Code	Texture	Colour	Elevation	Translucent	Gram	Shape	Oxidase	Indole	Catalase	Motility	Coagulase	Citrate	MR	VP	Glucose	Lactose	Maltose	Sucrose	Fluctose	Identified Probable Organism
A	Mucoid	Creamy	Raised	Opaque	+ve	Rod	+	-	+	+	-	+	+	-	+	-	-	-	-	*Bacillus* sp.
B	Moist	Yellow	Smooth	Translucent	+ve	Cocci	-	-	+	-	+	+	+	+	+	+	+	+	+	*Staphylococcus* sp
C	Moist	Creamy	Raised	Opaque	-ve	Rod	+	-	+	+	-	-	+	-	+	-	+	-	-	*Salmonella* sp.
D	Smooth	Clear	Raised	Translucent	-ve	Rod	-	-	+	-	-	-	+	-	+	-	-	+	-	*Shigella* sp.
E	Dried	Green	Flat	Opaque	-ve	Rod	+	+	+	+	-	+	+	-	+	-	+	+	-	*Pseudomonas* sp.
F	Moist	Clear	Raised	Opaque	-ve	Rod		-	+	+	-	-	+	-	+	-	+	+	+	*Enterobacter* sp.
G	Moist	Creamy	Raised	Translucent	-ve	Rod	+	+	+	+	-	-	+	-	+	+	+	+	-	*Escherichia coli*
H	Moist	Yellow	Raise	Translucent	+ve	Cocci	-	-	+	-	-	+	+	+	+	+	+	+	+	*Micrococcus* sp.
I	Dried	Milky	Flat	Opaque	-ve	Rod	-	-	+	+	+	-	+	-	+	+	+	+	-	*Acinetobacter* sp.
J	Moist	Pale	Raised	Opaque	-ve	Rod	-	-	+	+	+	-	+	-	+	+	+	+	-	*Klebsiella* sp.

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Discussion

Edible African walnuts (*Tetracarpidium conophorum*) sold as exposed cooked, unshelled nuts in Port Harcourt, Nigeria, were found to be contaminated with *Strongyloides stercoralis* and ten bacterial species. *Strongyloides stercoralis* is a geohelminth of wide distribution in stool samples of infected persons (Eze and Udugbo, 2017), as well as in soil (Olufotebi *et al*., 2019), vegetable (Robert *et al*., 2022) and fruit (Ezenwaka and Amuzie, 2021) samples in Nigeria. Infection with this parasite is known to be mostly asymptomatic (Greaves *et al*., 2013) but it can degenerate into a very severe disease, hyper-infection Strongyloidiasis in persons with impaired immune systems (Keiser and Nutman, 2020).

Total heterotrophic bacteria (THB) occur in animals and are often found in water samples. They are not pathogenic but may be opportunistic (Amanidaz *et al*., 2015). In water samples, the permissible level is 500 CFU/ml (Amanidaz *et al*., 2015). In the present study, the THB count was the highest with a value of $1.50x10^6$. Similarly, total faecal coliform are also naturally occurring, in animal digestive systems, in environmental samples (vegetation, soil etc) and are generally harmless. They are indicative of the presence of *Escherichia coli* which suggests contamination of samples by faecal matter (Schuerman, 2021). Faecal coliform counts are of more public health importance, especially if *E. coli* is present (Swistock and Sharpe, 2022). The total counts recorded in this present research for total coliform was $2.00x10^4$CFU/g while that for faecal coliforms was $1.30\ x10^4$CFU/g. High counts of faecal coliforms can cause diarrhea and fever in man (Swistock and Sharpe, 2022).

Unlike heterotrophic bacteria and total coliform, *Salmonella* and *Shigella* species are pathogenic causing Salmonellosis and Shigellosis respectively in man and they are diseases of public health importance (Shu-Kee *et al*., 2015; Strockbine *et al*., 2015). A value of $8.9\ x10^3$ CFU/g recorded in the African walnuts examined in this research is indicative of gasteroenteritis. In a research on the infection of food-handlers by intestinal parasites, *Salmonella* and *Shigella* in Ethiopia, Yesigat *et al*. (2020) reported *Strongyloides stercoralis*, *Salmonella* sp. and *Shigella* sp. at a prevalence of 4.5%, 2.5% and 1.6%, respectively. They reported significant association between untrimmed fingernails of food-handlers and prevalence of *Salmonella* and *Shigella* species. Food-handlers were identified as major carriers in the transmission of these enteric micro-organisms. Improper handling of food products, such as in preparation or storage, are also risk factors. As earlier stated, walnuts are exposed to touch by both the traders and buyers. The presence of *S. stercoralis* and *Salmonella*, *Shigella* as well as other bacteria hereby reported could have been through inadequate hygienic practices of the traders, buyers, and/or by the exposure of the nuts to environmental contaminations. *Staphylococcus* species, especially *S. aureus*, is responsible for a wide range of disease conditions in man, ranging from skin infections to respiratory tract infections and others. More disturbing about infection with these pathogens is the occurrence of antibiotic resistance in the strains (Cheung *et al*., 2020).

Other bacterial species isolated from the walnuts shells were *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Micrococcus* sp., *Acinetobacter* sp., and *Klebsiella* sp. *Bacillus* sp., *Enterobacter* sp. and *Klebsiella* sp. are coliform bacilli with some overt and opportunistic species that cause a variety of infectious diseases (Guentzel, 1996). Some species of *Pseudomonas* and *Acinetobacter* cause diseases that are "difficult to treat" (Sewunet *et al*., 2022). *Micrococcus* species are not pathogenic but have opportunistic strains (Nuñez, 2014). These parasitic and bacterial species isolated from the African walnuts examined in this research indicate that walnuts sold in unhygienic forms in the market are contaminated and should be thoroughly washed before consumption.

In conclusion, cooked, unshelled African walnuts sold in Port Harcourt were, found to be infested with the geohelminth *Strongyloides stercoralis* and contaminated with various bacterial species, most of which are pathogenic, especially *Salmonella* and *Shigella* species. Infection of these walnuts by these organisms is consequent of unhygienic handling. The manner in which they are marketed and handled predisposes them to infections from human contaminated fingers and other environmental samples.

It is therefore recommended that more hygienic means of storage and handling of these highly nutritious nuts should be enforced. On their parts, consumers should desist from the habit of cracking the nuts with their teeth as this creates a portal of entry for parasites and microorganisms.

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